

Improved Microtechnique for the Leptospiral Microscopic Agglutination Test¹

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A method for improving the original Galton microtechnique for detecting leptospiral antibodies has been developed. Simultaneous titrations were performed on 281 animal and human sera and 17 hyperimmune sera with the microscopic agglutination (MA) test and the improved microtechnique. Reproducibility of the improved microtechnique was determined independently on 65 animal sera by two laboratory sections. The results obtained by comparing positive test data from human and animal sera indicated that agreement between the original MA test and this new method exceeded 94%, whereas the original Galton microtechnique and the original MA test agreed in a maximum of 77% of the tests. This study indicates that the results obtained with the improved microtechnique are much more comparable to results obtained with the original MA test than are those obtained with the original Galton microtechnique.

The microscopic agglutination (MA) test is the recommended procedure for detecting leptospiral antibodies, measuring antibody titer and identifying unknown leptospiral isolates (1, 5). This procedure requires the use of tubes (1, 5) or plastic trays (4) for dilution and incubation, with the subsequent transfer of a drop of the antigen-serum mixture to a slide for reading agglutination. This procedure is tedious, time-consuming, and requires large quantities of antigen. In the original Galton microtechnique, dilutions are made and agglutination is observed directly in microdilution plates. A dark-field microscope equipped with a 3.5× objective and 10× oculars or a Zoom dissecting microscope is used. A major disadvantage of this technique is that only agglutinated leptospire can be observed.

The microtechnique reported here is similar to the one previously reported (2). Major differences are that plates with flat-bottom wells and a dark-field microscope equipped with a long-working-distance 10× objective, 10× oculars, and a dry, dark-field condenser are used. In a negative test, unagglutinated leptospire can be observed. The reliability of this improved microtechnique and comparative data with the tray method (4) are reported.

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MATERIALS AND METHODS

Microtechnique equipment. Equipment used was as follows: (i) disposable plastic microdilution plates with flat-bottom wells (Microtest II, Falcon Plastics, Oxnard, Calif., and Linbro Chemical Co., New Haven, Conn.); (ii) disposable microtiter pipettes equipped with a 0.025-ml or 0.05-ml dropper tip (Linbro Chemical Co., New Haven, Conn.); (iii) multimicrodiluter handle equipped with 0.025-ml or 0.05-ml microdiluters (Cooke Engineering Co., Alexandria, Va.); (iv) plate covers (Microtest II, Falcon Plastics, Oxnard, Calif., and Linbro Chemical Co., New Haven, Conn.).

Before use, plastic fibers or dust particles should be blown from the flat-bottom wells with a jet of clear air. The microdilution plates may be used repeatedly if they are washed immediately after each use with a sodium hypochlorite solution, rinsed in distilled water, and dried.

Sera. Diagnostic sera (281) from animal and human sources and 17 rabbit hyperimmune sera were tested simultaneously by the improved microtechnique and the tray method (4).

The following methods for preparing twofold dilutions of serum in the microdilution plates were used.

Diagnostic and Research Laboratories (DRL).

(i) One drop (0.025 ml) of phosphate-buffered saline (PBS) was placed in each well in the plate. (ii) One drop (0.025 ml) of a 1:50 serum dilution was added to wells in the first row.

Center for Disease Control (CDC). (i) One drop (0.05 ml) of PBS was placed in each well in the plate except for the wells in the first row. (ii) Two drops

(0.10 ml) of a 1:25 serum dilution were added to the first row of wells.

With the 0.025-ml (DRL) or 0.05-ml (CDC) microdiluter, the dilutions in the first row were mixed by twirling the diluters 10 to 15 times. Diluters were transferred to the next row and mixed. Mixing with the diluters was repeated for the desired number of dilutions. After the last dilution was mixed, diluters were rinsed by twirling them in distilled water and were then blotted dry.

Most contaminated sera can be cleared by centrifugation, but minor cloudiness will not affect the microtechnique.

Antigen. Antigens were 4- to 7-day-old cultures of leptospira grown in Stuart or polysorbate (EMJH) medium (Difco Laboratories, Detroit, Mich.). The antigen concentration was adjusted to 100 to 200 organisms per high-power field (450 \times), which is equivalent to a McFarland no. 0.5 or a Roesler 20 turbidity unit (3).

A separate 0.025-ml (DRL) or 0.05-ml (CDC) dropper was used for each antigen. One drop of antigen was added to each serum dilution. Each antigen was added in a separate column. The plates were gently shaken to mix contents, covered to exclude debris and prevent evaporation, and incubated at room temperature (25 to 30 C) for 2 h.

Reading of tests. The plates were placed on the stage of a dark-field microscope equipped with a long-working-distance, 10 \times objective (no. 599-003, E. Leitz, Inc., Rockleigh, N.J.) and 10 \times eye pieces, and the wells were examined for agglutination. The end point in a positive test was the highest dilution in which at least 50% of the leptospire were agglutinated.

RESULTS

Comparative data were obtained by testing diagnostic animal sera (DRL) and human, animal, and hyperimmune sera (CDC) by the improved microtechnique and by the MA tests.

We performed 390 comparative tests on 78 animal sera using five leptospiral antigens (DRL). Of these tests, 69 were positive and 316 were negative by both techniques, with an agreement of 98.7% (Table 1). A positive result was obtained by at least one method on 74 of the 390 tests. The titers obtained by the improved microtechnique were the same as those obtained by the MA technique in 55.4% (41/74) of these tests (Fig. 1). Titers were the same or within one dilution in 94.6% (70/74).

A similar evaluation was performed at CDC using 172 human diagnostic sera and 13 leptospiral antigens. Of the 1,337 comparative tests performed on these samples, 1,327 were classified by the two tests as positive or negative with an agreement of 99.3% (Table 2). Positive results were obtained by at least one method on 134 of the 1,337 tests. When the titers of these

TABLE 1. Comparative summary of results obtained from 390 tests on 78 unknown animal sera examined by the microscopic agglutination test and the microtechnique with leptospiral antigens at the Diagnostic and Research Laboratories^a

Improved microtechnique	Microscopic agglutination		Totals
	Positive	Negative	
Positive	69	4	73
Negative	1	316	317
Totals	70	320	390

^a Agreement on tests: 385/390 = 98.7%.

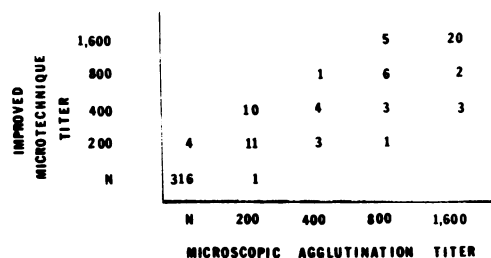


FIG. 1. Comparative reactions at the Diagnostic and Research Laboratories on 390 tests performed on 78 animal sera by microscopic agglutination and microtechnique with five leptospiral antigens. Using positive reactions only, tests with same reactions: 41/74 = 55.4% agreement; tests with same reaction or within one dilution: 70/74 = 94.6%.

TABLE 2. Comparative summary of results obtained from 1,337 tests on 172 unknown human sera examined by the microscopic agglutination test and the microtechnique with 13 leptospiral antigens at the Center for Disease Control^a

Improved microtechnique	Microscopic agglutination		Totals
	Positive	Negative	
Positive	124	10	134
Negative	0	1,203	1,203
Totals	124	1,213	1,337

^a Agreement on tests: 1,327/1,337 = 99.3%.

positive tests were plotted for comparison, the end points were the same in 58.9% (79/134) of the tests (Fig. 2). Titers were the same or within one dilution in 99.3% (133/134).

Thirty-one animal diagnostic sera were also evaluated with 13 leptospiral antigens at CDC. When both methods were used to compare 392 tests, 65 tests were positive and 314 were negative, with an agreement of 96.7% (Table 3).

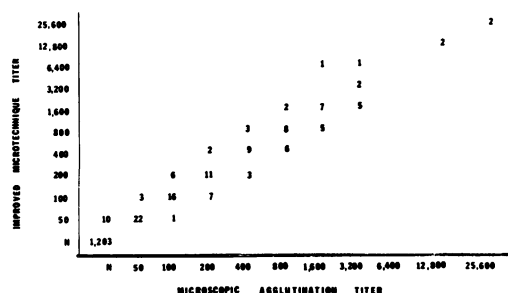


FIG. 2. Comparative reactions at the Center for Disease Control on 1,337 tests performed on 172 unknown human sera by microscopic agglutination and microtechnique with 13 leptospiral antigens. Using positive reactions only, tests with same reactions: $79/134 = 58.9\%$; tests with same reaction or within one dilution: $133/134 = 99.3\%$.

TABLE 3. Comparative summary of results obtained from 392 tests on 31 unknown animal sera examined by the microscopic agglutination test and the microtechnique with 13 leptospiral antigens at the Center for Disease Control^a

Improved microtechnique	Microscopic agglutination		Totals
	Positive	Negative	
Positive	65	13	78
Negative	0	314	314
Totals	65	327	392

^a Agreement on tests: $379/392 = 96.2\%$.

A positive result was obtained by at least one method on 78 of the 392 tests. When the titers of these positive tests were plotted for comparison, the end points were the same in 53.8% (42/78) of these tests (Fig. 3). Sample agreement was the same or within one dilution in 98.7% (77/78).

Comparative titers of 12 homologous sera tested at CDC with their respective antigens on 5 different days were within one dilution in 96.7% (58/60) of the tests. When five hyperimmune sera and 13 antigens were used, 98.5% (64/65) of the tests were in agreement within one dilution.

To determine reproducibility of this improved microtechnique, 65 animal sera were tested independently by two laboratory sections at CDC. When the titers of the 42 positive sera were plotted for comparison, end points were the same in 74.0% (71/96) of the tests (Fig. 4). Sample agreement was the same or within one dilution in 100% of the tests performed by the two sections.

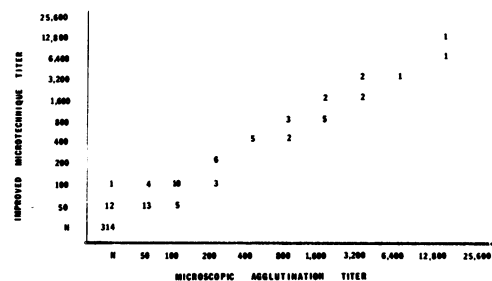


FIG. 3. Comparative reactions at the Center for Disease Control on 392 tests performed on 31 unknown animal sera by microscopic agglutination and microtechnique with 13 leptospiral antigens. Using positive reactions only, tests with same reactions: $42/78 = 53.8\%$; tests with same reaction or within one dilution: $77/78 = 98.7\%$.

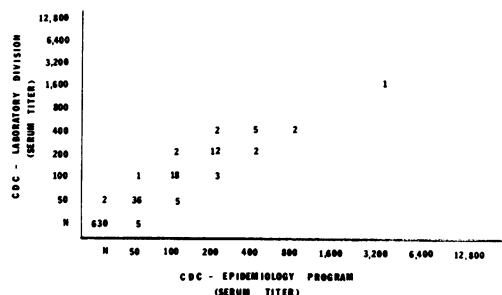


FIG. 4. Comparative reactions by two laboratory sections at the Center for Disease Control on 726 tests performed on 65 animal sera utilizing the microtechnique. Using positive reactions only, tests with same reactions: $71/96 = 74.0\%$; tests with same reaction or within one dilution: $96/96 = 100\%$.

DISCUSSION

In the current procedure for conducting the MA test, either tubes or plastic trays are used for dilution and incubation. This procedure requires large amounts of serum, antigen, and equipment. To observe the reaction, a drop of material must be transferred to a slide. The reading is clear because the unagglutinated leptospire can be observed. The dilution method and transfer of material to the slide greatly extends the time and labor required to perform the test. Moreover, there is a great chance of exposure to infection during the transfer of the antigen-serum mixture to the slide.

Using the microtechnique as a method to perform the MA test greatly reduced the amount of material and time required for the test. The microtechnique also made the test safer and easier to perform since the dilution, incubation, and reading were done in the mi-

crodilution plate. A disadvantage of the original microtechnique is that only the agglutinated leptospires can be observed (Fig 5). False negatives could be recorded since any wells in which

the antigen was not added would be read as negative.

The technique reported here combines the advantages of the original microtechnique with

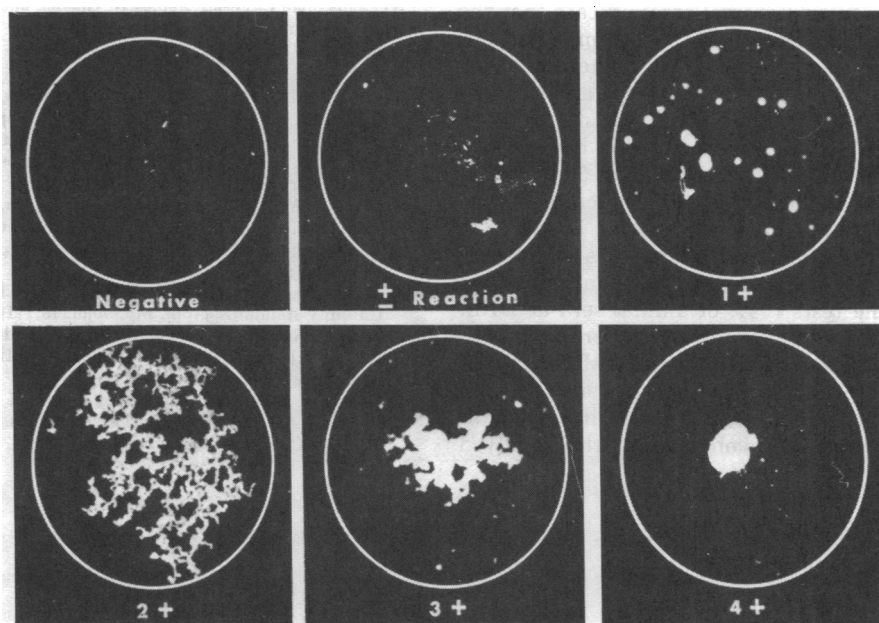


FIG. 5. *Leptospiral agglutination by Galton microtechnique; negative reaction and degrees of positive reactions.*

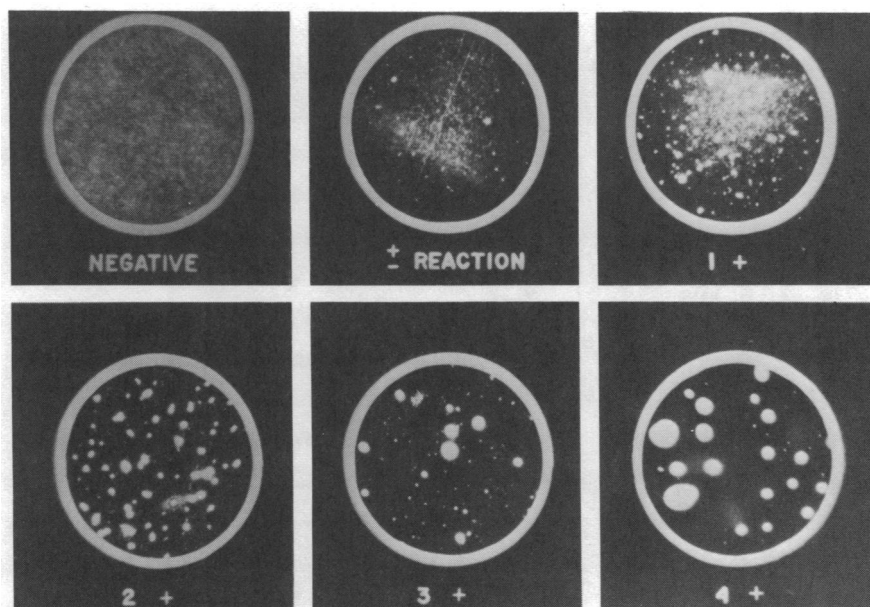


FIG. 6. *Leptospiral agglutination by improved microtechnique; negative reaction and degrees of positive reactions.*

the readability of the tube and tray method. The reaction observed in the flat-bottom well using the long-working-distance objective is comparable to that seen with a slide (Fig. 6).

The results obtained by comparing test data from human and animal sera indicate that the agreement between the MA and improved microtechnique exceeded 96%. Agreement between the original microtechnique and MA test was about 87% (2).

Identical titers were obtained by the two methods used in this evaluation in 54 to 60% of the tests, whereas the original microtechnique and MA test were in agreement about 43%. Positive titers obtained during this study were the same or within one dilution in more than 94% of the tests (94% of animal sera at DRL, and 99% of human and animal sera at CDC). The original microtechnique and MA test were comparable with 65% of the human sera and 77% of the animal sera. This evaluation indicates that the results obtained by the improved

microtechnique are much more comparable to the MA test than those obtained with the original microtechnique.

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